

EUKARYOTIC BIOSENSOR MAKING USE OF A CALCIUM REGULATED LIGHT EMITTING ENZYME1 **"Eukaryotic Biosensor"**

2

3 The present invention provides a method of using
4 transformed eukaryotic cells or organisms for
5 determining the presence or absence of at least one
6 toxic substance in a sample and for assisting in the
7 identification of the toxicant(s). More
8 specifically there is provided a toxicity assay for
9 various uses including determining the presence of
10 toxins, general cytotoxicity testing of pure
11 chemicals and chemical mixtures in particular for
12 drug development testing, testing of food and drink
13 products, cosmetics testing and identification of
14 organisms in particular of fungal strains

15

16 The release of contaminating substances into an
17 environment such as a waterway or an area of
18 agricultural land can have serious effects on the
19 ecosystems found in that environment. It is
20 important to be able to analyse these effects both
21 prior to the release of such contaminants so as to

1 manage their treatment or release, and after release
2 so as to determine and counteract their effects.

3
4 Current methods used to monitor water quality and
5 screen effluent generally involve chemical toxicity
6 tests. However, these tests require a general idea
7 of the type of contaminant being tested for and can
8 be very expensive.

9
10 Similarly the presence of contaminating substances
11 or toxins can be problematic in other areas such as
12 food and drink manufacture and cosmetics
13 manufacture. There are also instances, such as in
14 drug development and cosmetic industry, where the
15 substance of interest i.e. the potential new drug
16 may itself be a contaminating substance or toxin and
17 this needs to be checked.

18
19 Biosensors are used for toxicity testing and are
20 well known in the field. Toxicity depends on a
21 variety of factors including pH, temperature,
22 salinity and contaminant concentration, but depends
23 especially on the test organism used in the sensor.

24
25 One of the most commonly used organisms is the
26 bioluminescent bacterium, *Vibrio fischeri*. The
27 bioluminescence involved is mediated by the
28 luciferin-luciferase enzyme system wherein light
29 emission is dependent on the electron transfer
30 chain. Any disruption to the electron transfer
31 chain, for example on exposure to a toxicant,
32 affects light emission. Light emission at the time a

1 substance is added is therefore indicative of the
2 presence of a toxic substance.

3

4 This system, however, only provides a simple
5 indication of whether a contaminant is toxic or not.
6 No detailed information is obtained on how toxic the
7 contaminant is, nor is the contaminant identified.

8

9 The terms toxicant and toxin as herein described
10 relate to compounds, chemicals and mixtures of
11 chemicals which have an effect on eukaryotic cells
12 or organisms and in particular which are toxic to
13 eukaryotic organisms such as fungus or which have
14 anti-fungal activity.

15

16 The term eukaryote as herein described relates to
17 eukaryotic cells or organisms.

18

19 According to a first aspect of the present invention
20 there is provided a method of determining the
21 presence of a toxicant in a test sample, comprising
22 the steps of;

- 23 - exposing a eukaryote that has been
- 24 transformed with a light emitting Ca^{2+}
- 25 regulated photoprotein gene to a test sample
- 26 - measuring the light produced by the
- 27 transformed cell/organism
- 28 - determining whether the amount of light is
- 29 above or below a defined threshold at the
- 30 time of exposure.

31

32

1 Optionally the eukaryote is a fungi.
2 (throughout this document fungi should be considered
3 under its typical classification as covering both
4 multicellular organisms and unicellular organisms
5 such as the yeast *Saccharomyces cerviseae*
6 Preferably the fungi is a filamentous fungi.

7
8 More preferably the fungi is of the *Aspergillus*
9 species.

10
11
12 Alternatively the eukaryote is a mammalian cell.

13
14 A further alternative is that the eukaryote is a
15 plant cell.

16
17 Preferably the test sample comprises a toxicant.

18
19 Preferably the light emitting Ca^{2+} regulated
20 photoprotein gene is a recombinant gene.

21
22 Preferably the light emitting Ca^{2+} regulated
23 photoprotein gene is selected from the group
24 comprising;

- 25 - aequorin gene
- 26 - halistaurin (mitrocomin) gene
- 27 - phialidin (clytin) gene
- 28 - obelin gene
- 29 - mnemiopsin gene
- 30 - berovin gene

31

1 Optionally, the light emitting Ca^{2+} regulated
2 photoprotein gene may be a functional homologue of a
3 gene selected from the group comprising;

- 4 - aequorin gene
- 5 - halistaurin (mitrocomin) gene
- 6 - phialidin (clytin) gene
- 7 - obelin gene
- 8 - mnemiopsin gene
- 9 - berovin gene

10

11 Most preferably the light emitting Ca^{2+} regulated
12 photoprotein gene is an aequorin gene.

13

14 More preferably the light emitting Ca^{2+} regulated
15 photoprotein gene is a recombinant aequorin gene.

16

17 Preferably the light that is measured is in the form
18 of luminescence.

19

20 Optionally the test sample is added in advance of
21 the application of a stimulus to the test sample.

22

23 Preferably the stimulus is at least one or more from
24 the group comprising; mechanical perturbation, hypo-
25 osmotic shock, and change in external calcium
26 chloride concentration, temperature shock, pH shock.

27

28 Preferably the test sample is added 1 minute to 1
29 hour prior to the application of the stimulus.

30

31 More preferably the test sample is added 5 minutes
32 prior to the application of the stimulus.

1
2 More preferably the test sample is added 30 minutes
3 prior to the application of the stimulus.

4
5 According to a second aspect of the present
6 invention there is provided a method of determining
7 the presence of a toxicant in a test sample,
8 comprising the steps of;

- 9 - exposing a eukaryote that has been
10 transformed with a light emitting Ca^{2+}
11 regulated photoprotein gene to a test sample
- 12 - measuring the light produced by the
13 transformed cell/organism
- 14 - determining whether the amount of light is
15 above a defined threshold at a specified
16 time after the time of exposure.

17
18 Optionally the method comprises the step of
19 determining whether the amount of light is below a
20 defined threshold.

21
22 Optionally the specified time after the time of
23 exposure is 11 minutes.

24
25 Optionally the eukaryote is a fungi.

26
27 Preferably the fungi is a filamentous fungi.

28
29 More preferably the fungi is of the *Aspergillus*
30 species.

31
32

1 Alternatively the eukaryote is a mammalian cell.

2

3 A further alternative is that the eukaryote is a
4 plant cell.

5

6 Preferably the test sample comprises a toxicant.

7

8 Preferably the light emitting Ca^{2+} regulated
9 photoprotein gene is a recombinant gene.

10

11 Preferably the light emitting Ca^{2+} regulated
12 photoprotein gene is selected from the group
13 comprising;

- 14 - aequorin gene
- 15 - halistaurin (mitrocomin) gene
- 16 - phialidin (clytin) gene
- 17 - obelin gene
- 18 - mnemiopsin gene
- 19 - berovin gene

20

21 Optionally, the light emitting Ca^{2+} regulated
22 photoprotein gene may be a functional homologue of a
23 gene selected from the group comprising;

- 24 - aequorin gene
- 25 - halistaurin (mitrocomin) gene
- 26 - phialidin (clytin) gene
- 27 - obelin gene
- 28 - mnemiopsin gene
- 29 - berovin gene

30

1 Most preferably the light emitting Ca^{2+} regulated
2 photoprotein gene is an aequorin gene.

3

4 More preferably the light emitting Ca^{2+} regulated
5 photoprotein gene is a recombinant aequorin gene.

6

7 Preferably the light that is measured is in the form
8 of luminescence.

9

10 Optionally the test sample is added in advance of
11 the application of a stimulus to the test sample.

12

13 Preferably the stimulus is at least one or more from
14 the group comprising; mechanical perturbation, hypo-
15 osmotic shock, change in external calcium chloride
16 concentration, temperature shock, pH shock.

17

18 Preferably the test sample is added 1 minute to 1
19 hour prior to the application of the stimulus.

20

21 More preferably the test sample is added 5 minutes
22 prior to the application of the stimulus.

23

24 More preferably the test sample is added 30 minutes
25 prior to the application of the stimulus.

26

27 According to a third aspect of the present invention
28 there is provided a method of determining the
29 presence of a toxicant in a test sample, comprising
30 the steps of;

- 1 - exposing a eukaryote that has been
- 2 transformed with a light emitting Ca^{2+}
- 3 regulated photoprotein gene to a test sample
- 4 - measuring the light produced by the
- 5 transformed cell/organism
- 6 - and comparing at least one parameter of the
- 7 light measurement data with a bank of known
- 8 toxicity reference data.

9

10 Optionally the method comprises the step of
11 determining whether the amount of light is below a
12 defined threshold.

13

14 Optionally the specified time after the time of
15 exposure is 11 minutes.

16

17 Optionally the eukaryote is a fungi.

18

19 Preferably the fungi is a filamentous fungi.

20

21 More preferably the fungi is of the *Aspergillus*
22 species.

23 Do we need next two sentences

24 Most preferably the fungi is *Aspergillus awamori*.

25

26 Most preferably the strain of *Aspergillus awamori* is
27 strain 66A.

28

29 Alternatively the eukaryote is a mammalian cell.

30

31 A further alternative is that the eukaryote is a
32 plant cell.

1

2 Preferably the test sample comprises a toxicant.

3

4 Preferably the light emitting Ca^{2+} regulated
5 photoprotein gene is a recombinant gene.

6

7 Preferably the light emitting Ca^{2+} regulated
8 photoprotein gene is selected from the group
9 comprising;

- 10 - aequorin gene
- 11 - halistaurin (mitrocomin) gene
- 12 - phialidin (clytin) gene
- 13 - obelin gene
- 14 - mnemiopsin gene
- 15 - berovin gene

16

17 Optionally, the light emitting Ca^{2+} regulated
18 photoprotein gene may be a functional homologue of a
19 gene selected from the group comprising;

- 20 - aequorin gene
- 21 - halistaurin (mitrocomin) gene
- 22 - phialidin (clytin) gene
- 23 - obelin gene
- 24 - mnemiopsin gene
- 25 - berovin gene

26

27 Most preferably the light emitting Ca^{2+} regulated
28 photoprotein gene is an aequorin gene.

29

30 More preferably the light emitting Ca^{2+} regulated
31 photoprotein gene is a recombinant aequorin gene.

1
2 Preferably the light that is measured is in the form
3 of luminescence.
4
5 Optionally the test sample is added in advance of
6 the application of a stimulus to the test sample.
7
8 Preferably the stimulus is at least one or more from
9 the group comprising; mechanical perturbation, hypo-
10 osmotic shock, change in external calcium chloride
11 concentration, temperature shock, pH shock.
12
13 Preferably the test sample is added 1 minute to 1
14 hour prior to the application of the stimulus.
15
16 More preferably the test sample is added 5 minutes
17 prior to the application of the stimulus.
18
19 More preferably the test sample is added 30 minutes
20 prior to the application of the stimulus.
21
22 Preferably, the method is used to determine the
23 amount of toxicant in the sample.
24
25 Optionally, the method is used to identify the
26 toxicant in the sample.
27
28 According to a fourth aspect of the present
29 invention there is provided a method of determining
30 the presence of a toxicant in a test sample,
31 comprising the steps of;

- 1 - exposing a eukaryote that has been
- 2 transformed with a light emitting Ca^{2+}
- 3 regulated photoprotein gene to a test sample
- 4 - measuring the light produced by the
- 5 transformed cell/organism
- 6 - converting the light data into a cytosolic
- 7 free calcium ion concentration trace,
- 8 - and comparing at least one parameter of the
- 9 cytosolic free calcium ion concentration
- 10 trace with a bank of known toxicity
- 11 reference data.

12

13 Optionally the method comprises the step of
14 determining whether the amount of light is below a
15 defined threshold.

16

17 Optionally the specified time after the time of
18 exposure is 11 minutes.

19

20 Optionally the eukaryote is a fungi.

21

22 Preferably the fungi is a filamentous fungi.

23

24 More preferably the fungi is of the *Aspergillus*
25 species.

26

27

28 Alternatively the eukaryote is a mammalian cell.

29

30 A further alternative is that the eukaryote is a
31 plant cell.

32

1 Preferably the test sample comprises a toxicant.

2

3 Preferably the light emitting Ca^{2+} regulated
4 photoprotein gene is a recombinant gene.

5

6 Preferably the light emitting Ca^{2+} regulated
7 photoprotein gene is selected from the group
8 comprising;

9

- aequorin gene

10

- halistaurin (mitrocomin) gene

11

- phialidin (clytin) gene

12

- obelin gene

13

- mnemiopsin gene

14

- berovin gene

15

16 Optionally, the light emitting Ca^{2+} regulated
17 photoprotein gene may be a functional homologue of a
18 gene selected from the group comprising;

19

- aequorin gene

20

- halistaurin (mitrocomin) gene

21

- phialidin (clytin) gene

22

- obelin gene

23

- mnemiopsin gene

24

- berovin gene

25

26 Most preferably the light emitting Ca^{2+} regulated
27 photoprotein gene is an aequorin gene.

28

29 More preferably the light emitting Ca^{2+} regulated
30 photoprotein gene is a recombinant aequorin gene.

31

1 Preferably the light that is measured is in the form
2 of luminescence.

3

4 Optionally the test sample is added in advance of
5 the application of a stimulus to the test sample.

6

7 Preferably the stimulus is at least one or more from
8 the group comprising; mechanical perturbation, hypo-
9 osmotic shock, change in external calcium chloride
10 concentration, temperature shock, pH shock.

11

12 Preferably the test sample is added 1 minute to 1
13 hour prior to the application of the stimulus.

14

15 More preferably the test sample is added 5 minutes
16 prior to the application of the stimulus.

17

18 More preferably the test sample is added 30 minutes
19 prior to the application of the stimulus.

20 Preferably light is measured for between 1 minute
21 and 5 hours following the application of the
22 stimulus.

23

24 More preferably light is measured for 5 minutes
25 following the application of the stimulus.

26

27 Preferably, the cytosolic free calcium ion trace is
28 a plot of the cytosolic free calcium ion
29 concentration against time.

30

31 Preferably the parameter is at least one or more
32 selected from the group comprising;

- 1 - lag time
- 2 - rise time
- 3 - absolute amplitude
- 4 - relative amplitude
- 5 - Length of transient
- 6 - number of cytosolic free calcium ion
- 7 concentration increases
- 8 - percentage increase in final cytosolic free
- 9 calcium ion concentration resting level
- 10 - percentage increase in recovery time
- 11 - percentage increase in pre-stimulating
- 12 cytosolic free calcium ion concentration
- 13 resting level
- 14 - Total concentration of Ca^{2+} released.

15

16 Preferably, the method is used to determine the
17 amount of toxicant in the sample.

18

19 Optionally, the method is used to identify the
20 toxicant in the sample.

21

22 According to a fifth aspect of the present invention
23 there is provided an assay for use in determining
24 the presence of a known toxicant in a test sample,
25 the assay comprising the steps of;

- 26 - exposing a fungi transformed with a
- 27 recombinant aequorin gene to a test sample of
- 28 a substance,
- 29
- 30 - measuring the luminescence produced by the
- 31 fungi,

1

2

- converting the luminescence data into a
cytosolic free calcium ion concentration
trace,

5

6

- and comparing at least one parameter of the
cytosolic free calcium ion concentration
trace with a bank of known toxicity reference
data.

9

10

Preferably the cytosolic free calcium ion trace is a
plot of the cytosolic free calcium ion concentration
against time.

14

Preferably the fungi transformed with a recombinant
aequorin gene is a filamentous fungi.

17

More preferably the fungi is of the *Aspergillus*
species.

20

21

Preferably the substance is a contaminant.

23

Preferably the substance is a contaminated sample.

25

Preferably the parameter is at least one or more
selected from the group comprising; lag time, rise
time, absolute amplitude, relative amplitude, length
of transient at 20%, 50% and 80% of maximum
amplitude , number of cytosolic free calcium ion
concentration increases, percentage increase in
final cytosolic free calcium ion concentration

32

1 resting level, percentage increase in recovery time
2 and percentage increase in the total amount of Ca^{2+}
3 released.

4
5 Optionally, the test sample is added in advance of
6 the application of a stimulus to the test sample.

7
8 Preferably the stimulus is at least one or more from
9 the group comprising; mechanical perturbation, hypo-
10 osmotic shock, change in external calcium chloride
11 concentration, temperature shock and pH shock.

12
13 Preferably the test sample is added 1 minute to 1
14 hour prior to the application of the stimulus.

15
16 More preferably the test sample is added 5 minutes
17 prior to the application of the stimulus.

18
19 More preferably the test sample is added 30 minutes
20 prior to the application of the stimulus.

21
22 In such instances, the parameters may include at
23 least one or more selected from the group
24 comprising; lag time, rise time, absolute amplitude,
25 relative amplitude Length of transient at 20%, 50%
26 and 80% of maximum amplitude, number of cytosolic
27 free calcium ion concentration increases, percentage
28 increase in final cytosolic free calcium ion
29 concentration resting level, percentage increase in
30 recovery time, percentage increase in pre-
31 stimulating cytosolic free calcium ion concentration

1 resting level and percentage increase in the total
2 amount of Ca^{2+} released.

3

4 Preferably luminescence is measured for between 1
5 minute and 5 hours following the application of the
6 stimulus.

7

8 More preferably luminescence is measured for 5
9 minutes following the application of the stimulus.

10

11 Preferably, the method is used to determine the
12 amount of toxicant in the sample.

13

14 Optionally, the method is used to identify the
15 toxicant in the sample.

16

17 In order to further explain the present invention
18 details of a number of experiments are provided.

19

20 A first experiment comprises testing the effect of
21 pre-incubation of *Aspergillus awamori* with toxicants
22 on cytosolic free calcium ion concentration response
23 to an increase in external calcium chloride.

24

25 A further set of experiments described herein shows
26 attempts to obtain characteristic data for a range
27 of different toxicants at a number of different
28 concentrations. The results demonstrate that each
29 toxicant at each concentration produces a
30 distinctive cytosolic free calcium ion concentration
31 trace whose traits could be used to identify and
32 characterise a toxicant present in a test sample.

1
2 A final experiment attempts to determine whether it
3 is possible to identify and characterise individual
4 toxicants from testing samples of mixtures of
5 toxicants in different proportions. The traces
6 produced are distinct for each mixture.

7
8 These results show that it is possible to
9 characterise and identify a specific toxicant from a
10 test sample by using the characteristic data
11 obtained from a cytosolic free calcium ion
12 concentration trace.

13
14 It is also possible to characterise and identify a
15 specific toxicant from a test sample by using the
16 characteristic data obtained from light readings.
17 The main difference between doing light emission and
18 cytosolic free calcium ion concentrations is the
19 removing the step of converting the luminescence
20 data into a cytosolic free calcium ion concentration
21 trace".

22
23 So the method is:

24
25 An assay for use in determining the presence of a
26 known toxicant in a test sample, the assay
27 comprising the steps of;

- 28 - exposing a fungi transformed with a
29 recombinant aequorin gene to a test sample of
30 a substance,
31 - measuring the luminescence produced by the
32 fungi in relative light units (RLU),

1 - and calculating the following parameters: lag
2 time, rise time, length of transient
3 (LT_{20} , LT_{50} , LT_{80}), absolute amplitude, relative
4 amplitude, recover time, final level of
5 luminescence, initial level of luminescence,
6 total luminescence.

7
8 Since RLU are not normalised with regard to the
9 biomass, the parameters measured in relative light
10 units (RLU) are different from the cytosolic free
11 calcium ion concentration $[Ca^{2+}]$. Figs 24 and 25 show
12 that the decrease in amplitude caused by 260 mg/l
13 Cr^{6+} is 75% in RLU, and only 65% in Ca^{2+}
14 concentration. Other parameters would differ in a
15 similar way.

16
17 Most of the toxicity testing for environmental
18 pollutants is usually carried out using RLU and
19 therefore the light-emitting assay would be
20 particularly helpful if used alongside other
21 existing biosensors.

22
23 The parameters referred to herein relate to the
24 following;

25
26 Lag Time, the time from addition of the test sample
27 to the time when the cytosolic free calcium ion
28 concentration, $[Ca^{2+}]_c$, began to rise;

29
30 Rise Time, the time from addition of the test sample
31 to the time at which maximum $[Ca^{2+}]_c$ was reached;
32

1

2

3 Number of $[Ca^{2+}]_c$ Rises, the number of peaks in
4 $[Ca^{2+}]_c$;

5

6 Percentage Increase in Final $[Ca^{2+}]_c$ Resting Level,
7 the percentage increase in resting $[Ca^{2+}]_c$ at the end
8 of the experiment, where the control value is taken
9 to be 100%;

10

11 Percentage Increase in Recovery Time, percentage
12 increase in recovery time where recovery time
13 represents the total amount of $[Ca^{2+}]_c$ released
14 during the period of time from the point when the
15 maximum amplitude following calcium chloride
16 treatment was achieved to the point when the $[Ca^{2+}]_c$
17 reached its final resting level. Recovery time was
18 initially calculated for control cultures. In the
19 control this period of time was calculated as 250
20 seconds. For the cultures subjected to the
21 treatment with toxicant(s) the total amount of
22 $[Ca^{2+}]_c$ was calculated for the same period of 250
23 seconds starting from the maximum amplitude. The
24 recovery time of the control cultures was therefore:

25

26 total amount of $[Ca^{2+}]_c$ (μM) for the toxicant-treated
27 samples over 250 seconds x 100

28 total amount of $[Ca^{2+}]_c$ (μM) for the control sample
29 over 250 seconds

30

31 Percentage Increase in pre-Stimulating $[Ca^{2+}]_c$
32 Resting Level, the percentage increase in $[Ca^{2+}]_c$

1 prior to the stimulus, where the control value is
2 taken to be 100%.

3

4 Percentage change in total amount of calcium
5 released during the transient at stage 1 -
6 calculated by integration of the all luminescence
7 obtained after addition of the compounds of interest
8 before subsequent stimulation with physico-chemical
9 stimuli.

10

11 Percentage change in total amount of calcium
12 released during the transient at stage 2 -
13 calculated by integration of the all luminescence
14 obtained after the fungus is stimulated with one of
15 the physico-chemical stimuli.

16

17 Percentage change in total amount of calcium
18 released during the whole transient - calculated by
19 integration of the all luminescence obtained during
20 the period of experiment.

21

22 Length of transient (LT) - this parameter describes
23 the length of the transient when the amplitude of
24 the response is equal a certain percentage from the
25 maximum amplitude.

26 LT₂₀ (Length of transient at Amplitude=20% of maximum
27 Amplitude)

28 LT₅₀ (Length of transient at Amplitude=50% of maximum
29 Amplitude)

30 LT₈₀ (Length of transient at Amplitude=80% of maximum
31 Amplitude)

32

1 All secondary increases have to be analysed by the
2 same parameters as primary increases during stages 1
3 and 2.

4 E.g. Amplitude, length, rise time, lag time,
5

6 Percentage change in amplitude should be assessed as
7 the absolute value from point 0 (A_a) and as the
8 relative value from the initial resting level (A_r).
9 The relative changes assess the ability of of the
10 eukaryote to respond to the physiological stimuli.

11 This parameter is important to assess the
12 physiological state of the eukaryote.

13 There is also the possibility of combining one or
14 more of these parameters to obtain further values
15 which can be used for identification of the
16 toxicants in the mixture. For example, the
17 summation of amplitude and recovery time will give
18 the value of total cytosolic free calcium ions
19 emitted from the time when $[Ca^{2+}]_c$ reaches its peak.
20 Also summation of lag time and rise time will give
21 the total time required for $[Ca^{2+}]_c$ to reach its
22 peak. The division of final $[Ca^{2+}]_c$ resting level
23 onto the pre-stimulation $[Ca^{2+}]_c$ resting level will
24 show how many times the $[Ca^{2+}]_c$ resting level has
25 changed after stimulation. Similarly, a division of
26 the final $[Ca^{2+}]_c$ resting level onto the initial
27 $[Ca^{2+}]_c$ resting level prior to the addition of
28 toxicant(s) gives further identifying data.

29 Additionally, the summation of all the data points
30 of the trace gives the total amount of cytosolic
31 free calcium ions released during the monitoring
32 period.

1 As mammalian cells are more complex than other
2 eukaryotes such as fungi or plants typically more
3 parameters will be considered.

4
5 The present invention will now be described with
6 reference to the following non-limiting examples and
7 with reference to the figures, wherein:

8
9 Figure 1 shows the characteristic $[Ca^{2+}]_c$ trace
10 produced on addition of 5mM external $CaCl_2$,
11 following a 5 minute pre-incubation with
12 different concentrations of 3,5-DCP.

13
14 Figure 2 shows the characteristic $[Ca^{2+}]_c$ trace
15 produced on addition of 5mM external $CaCl_2$,
16 following a 5 minute pre-incubation with
17 different concentrations of Cr^{6+} .

18
19 Figure 3 shows the characteristic $[Ca^{2+}]_c$ trace
20 produced on addition of 5mM external $CaCl_2$,
21 following a 5 minute pre-incubation with
22 different concentrations of Zn^{2+} .

23
24 Figure 4 shows the characteristic $[Ca^{2+}]_c$ trace
25 produced on addition of 5mM external $CaCl_2$,
26 following a 30 minute pre-incubation with
27 different concentrations of 3,5-DCP.

28
29 Figure 5 shows the characteristic $[Ca^{2+}]_c$ trace
30 produced on addition of 5mM external $CaCl_2$,

1 following a 30 minute pre-incubation with
2 different concentrations of Cr^{6+} .

3
4 Figure 6 shows the characteristic $[\text{Ca}^{2+}]_c$ trace
5 produced on addition of 5mM external CaCl_2 ,
6 following a 30 minute pre-incubation with
7 different concentrations of Zn^{2+} .

8
9 Figure 7 shows the characteristic cytosolic free
10 calcium ion concentration, $[\text{Ca}^{2+}]_c$, trace
11 produced on addition of 5mM CaCl_2 following a 5
12 minute pre-incubation with different
13 concentrations of 3,5-dichlorophenol, 3,5-DCP.

14
15 Figure 8 shows the characteristic $[\text{Ca}^{2+}]_c$ trace
16 produced on addition of 5mM CaCl_2 , following a
17 30 minute pre-incubation with different
18 concentrations of 3,5-DCP.

19
20 Figure 9 shows the characteristic $[\text{Ca}^{2+}]_c$ trace
21 produced on addition of 5mM CaCl_2 , following a 5
22 minute pre-incubation with different
23 concentrations of chromium ions, Cr^{6+} .

24
25 Figure 10 shows the characteristic $[\text{Ca}^{2+}]_c$ trace
26 produced on addition of 5mM CaCl_2 , following a
27 30 minute pre-incubation with different
28 concentrations of chromium ions, Cr^{6+} .

29
30 Figure 11 shows the characteristic $[\text{Ca}^{2+}]_c$ trace
31 produced on addition of 5mM CaCl_2 , following a 5

1 minute pre-incubation with different
2 concentrations of zinc ions, Zn^{2+} .

3

4 Figure 12 shows the characteristic $[\text{Ca}^{2+}]_c$ trace
5 produced on addition of 5mM CaCl_2 , following a
6 30 minute pre-incubation with different
7 concentrations of zinc ions, Zn^{2+} .

8

9 Figure 13 shows the values of $[\text{Ca}^{2+}]_c$ trace
10 parameters characteristic for different
11 concentrations of pentochlorophenol, PCP; sodium
12 dodecyl sulphate, SDS; and Toluene. Parameters
13 assessed are Lag Time, LT; Rise Time, RT;
14 Amplitude, A; Length of transient, LT50;
15 Percentage Increase in pre-Stimulating $[\text{Ca}^{2+}]_c$
16 Resting Level, %IpreSRL; Percentage Increase in
17 Final $[\text{Ca}^{2+}]_c$ Resting Level, %IFRL; Percentage
18 Increase in Recovery Time, %IRT; and Number of
19 $[\text{Ca}^{2+}]_c$ Increases.

20

21 Figure 14 shows the values of $[\text{Ca}^{2+}]_c$ trace
22 parameters characteristic for 3,5-DCP, PCP, Zn^{2+} ,
23 Cr^{6+} , Toluene, and SDS. Parameters assessed are
24 Lag Time, LT; Rise Time, RT; Amplitude, A;
25 Length of transient, LT50; Percentage Increase
26 in pre-Stimulating $[\text{Ca}^{2+}]_c$ Resting Level,
27 %IpreSRL; Percentage Increase in Final $[\text{Ca}^{2+}]_c$
28 Resting Level, %IFRL; Percentage Increase in
29 Recovery Time, %IRT; and Number of $[\text{Ca}^{2+}]_c$
30 Increases.

31

1 Figure 15 shows the values of $[Ca^{2+}]_c$ trace
2 parameters characteristic for different mixtures
3 of toxicants. Parameters assessed are Lag Time,
4 LT; Rise Time, RT; Amplitude, A; Length of
5 transient, LT50; Percentage Increase in pre-
6 Stimulating $[Ca^{2+}]_c$ Resting Level, %IpreSRL;
7 Percentage Increase in Final $[Ca^{2+}]_c$ Resting
8 Level, %IFRL; Percentage Increase in Recovery
9 Time, %IRT; and Number of $[Ca^{2+}]_c$ Increases.

10

11 Effect of pre-incubation of *Aspergillus awamori* with
12 toxicants on $[Ca^{2+}]_c$ response to external calcium
13 chloride

14

15 12 ml of sterile VS medium was inoculated with $1 \times$
16 10^5 spores per ml *A. awamori* strain 66A. 100 μ l of
17 the inoculated medium was added to each well of a
18 96-well plate and cultured in a humidity chamber in
19 the presence of free water at 30 °C for 24 hours.

20

21 The following toxicants were tested: 3,5-
22 dichlorophenol, zinc sulphate, and potassium
23 dichromate. Each toxicant was added in a total
24 volume of 25 μ l VS medium or water 5 or 30 minutes
25 before addition of 5 mM calcium chloride.

26

27 Luminescence was monitored for 5 minutes following
28 addition of $CaCl_2$. Aequorin was completely
29 discharged by adding 3M calcium chloride in 20%
30 ethanol. The total concentration is thus 1.5 M
31 calcium chloride in 10% ethanol.

32

1 Luminometry was performed using an EG & G Berthold
2 (Bad Wildbad, Germany) LB96P Microlumat luminometer.
3 Luminescence data was converted from real light
4 units to $[Ca^{2+}]_c$ values using the following equation:

5

$$6 \quad PCa = 0.332588 (-\log k) + 5.5593,$$

7

8 where k = luminescence counts per second/total
9 luminescence counts. Total luminescence is measured
10 as an integral of all luminescence up to complete
11 aequorin discharge.

12

13 The Equation is first described in Fricker, M.D.,
14 Plieth, C., Knight, H., Blancaflor, E., Knight,
15 M.R., White, N.S., and Gilroy, S. 1999. Fluorescence
16 and Luminescence Techniques to Probe Ion Activities
17 in Living Plant Cells. In Mason, W.T., editor,
18 *Fluorescent and Luminescent Probes*. Academic Press.
19 London. pp. 569-596.

20 The following parameters were assessed:

21 Rise Time, Amplitude, Length of transient, LT50 and
22 Final $[Ca^{2+}]_c$ Resting Level.

23

24 **Effects of different concentrations of toxicants on**
25 **$[Ca^{2+}]_c$ traces**

26

27 *Aspergillus awamori* were transformed with an
28 expression vector (pAEQ1-15) comprising a gene for
29 synthetic apoaequorin (*aeqS*) under the control of
30 the constitutive glucose-6-phosphate dehydrogenase
31 promoter (*gpdA*).

32

1 These transformants were cultured in 100 μ l of
2 Vogel's medium with 1% sucrose (VS medium) in
3 microwell plates for 24 hours before addition of a
4 toxicant or a control of distilled water. Toxicants
5 were dissolved in water to give the concentrations
6 shown below. 25 μ l of the each of the following
7 concentrations were added to each culture:
8

TOXICANT	CONCENTRATIONS (mg/l)
3,5-dichlorophenol (3,5-DCP)	0.112, 11.2, 112
Chromium ions (Cr^{6+})	15, 120, 260
Zinc ions (Zn^{2+})	180, 350, 700, 1300

9
10 The cultures were incubated for 5 or 30 minutes
11 before addition of 100 μ l 5mM CaCl_2 . Luminescence
12 was measured for 5 minutes using a plate
13 luminometer. Luminescence data was manually
14 converted from relative light units to cytosolic
15 free calcium ion concentration, $[\text{Ca}^{2+}]_c$. This was
16 then plotted against time and parameters of this
17 trace were analysed. Parameters assessed were as
18 follows:
19

20 Rise Time, the time from addition of CaCl_2 to the
21 moment when maximum $[\text{Ca}^{2+}]_c$ was achieved;
22 Amplitude, the maximum $[\text{Ca}^{2+}]_c$ reached during the
23 experiment;
24 Length of transient, at 50% of maximum amplitude the
25 width of the transient at the point where the
26 amplitude equals half of the maximum amplitude of
27 the transient;

1 and Final Resting $[Ca^{2+}]_c$ Level, the resting $[Ca^{2+}]_c$
2 at the end of the experiment.

3

4 Effects of further toxicants on $[Ca^{2+}]_c$ traces

5

6 Cultures of *Aspergillus awamori* as described above
7 were used to test the effects of further toxicants.
8 The concentrations of toxicants tested were made up
9 as follows in water, where the concentrations tested
10 were based on Dutch target and intervention values
11 for toxicants and Kelly Guidelines for the
12 classification of contaminated soils:

13

TOXICANT	CONCENTRATION (mg/l)
Pentochlorophenol, PCP	0.01, 0.1, 1, 5, 10
Sodium dodecyl sulphate, SDS	1, 10, 50, 100, 500
Toluene	1, 25
3,5-DCP	10
Zn^{2+}	700
Cr^{6+}	15

14

15

16 In the first set-up (S1), 100 μ l of each toxicant
17 concentration or of the control (VS medium) were
18 added to the cultures through built-in injectors and
19 luminescence monitored for 5 minutes. In a second
20 set of experiments (S2), cultures were pre-incubated
21 with the toxicant or control for 5 minutes before
22 addition of 5mM $CaCl_2$ in a total volume of 25 μ l
23 distilled water (pre-incubation can be anywhere
24 between 1 minute and 96 hours). Luminescence was

1 monitored for 5 minutes following addition of CaCl_2 .
2 (monitoring can be anywhere between 1 minute and 96
3 hours). Luminescence data was converted from
4 relative light units to $[\text{Ca}^{2+}]_c$ values as described
5 above. The following parameters were assessed in
6 S1:
7 Lag Time, the time from addition of CaCl_2 to the
8 time when $[\text{Ca}^{2+}]_c$ began to rise;
9 Rise Time;
10 Absolute amplitude;
11 Relative amplitude
12 Length of transient (LT20, LT50, LT80);
13 Percentage Increase in Final $[\text{Ca}^{2+}]_c$ Resting Level,
14 where the control value was taken to be 100%;
15 Percentage Increase in Recovery Time, where the
16 control value was taken to be 100%;
17 and Number of $[\text{Ca}^{2+}]_c$ Increases, the number of $[\text{Ca}^{2+}]_c$
18 transients.
19 Total Ca^{2+} concentration
20
21 In S2, the Percentage Increase in pre-Stimulating
22 $[\text{Ca}^{2+}]_c$ Resting Level, where the control value was
23 taken to be 100%, was assessed in addition to all of
24 the parameters tested in S1.

25

26 **Effects of mixtures containing different proportions**
27 **of toxicants on $[\text{Ca}^{2+}]_c$ traces**

28

29 The experiments described when examining the effects
30 of further toxicants were repeated for different
31 mixtures of toxicants. The following mixtures were
32 made up in water for testing:

1

2 6 mg/l 3,5-DCP + 12 mg/l Cr^{6+} 3 30 mg/l Cr^{6+} + 350 mg/l Zn^{2+} 4 10 mg/l 3,5-DCP + 350 mg/l Zn^{2+} 5 6 mg/l 3,5-DCP + 12 mg/l Cr^{6+} + 350 mg/l Zn^{2+}

6 Mixture 1: 20 mg/l Cadmium

7 100 mg/l Copper

8 50 mg/l Chromium

9 250 mg/l Zinc

10 500 mg/l SDS

11 Mixture 2: 20 mg/l Cadmium

12 100 mg/l Copper

13 50 mg/l Chromium

14 250 mg/l Zinc

15

16 These experiments demonstrate a novel finding that
17 each toxicant results in a different and
18 characteristic $[\text{Ca}^{2+}]_c$ transient. Additionally each
19 concentration of toxicant produces a unique $[\text{Ca}^{2+}]_c$
20 transient. From these characteristic fingerprint
21 responses a profile of data can be built up and used
22 to create a bank of data for each toxicant. Results
23 from testing samples can be compared with this data
24 bank and the presence of a particular toxicant can
25 thus be determined. Furthermore, details such as
26 the mode of action of the toxicant, and the amount
27 of toxicant present can be deduced from a comparison
28 with the bank of pre-gathered data.

29

30 Examples of types of testing that can be carried out
31 according to the present invention

32

1 Specific examples of types of test that can be
2 carried out according to the present invention are
3 given below. Although the tests below describe the
4 use of aequorin expressed fungi according to the
5 present invention, it can be seen that any
6 appropriate eukaryotic cell or organism could be
7 used (i.e. mammalian cells in place of the fungi)
8 which has been transformed with any appropriate gene
9 according to the present invention (i.e. halistaurin
10 in place of aequorin)

11

12 The examples refer to the following figures in
13 which;

14

15 Figure 16 shows a graph indicating the effect of 6
16 environmental samples on $[Ca^{2+}]_c$;

17

18 Figure 17 shows a graph indicating the effect of
19 ibuprofen analogue on $[Ca^{2+}]_c$;

20

21 Figure 18 shows a graph indicating the effect of
22 verpamil on $[Ca^{2+}]_c$;

23

24 Figure 19 is a table summarising the profiles of the
25 ibuprofenTM ((S)-(-)- o-Acetulmandelic acid) and
26 verapamilTM (Verapamil hydrochloride) analogues;

27

28 Figure 20 is a table summarising profiles of
29 cyclopiazonic acid (CPA) and KP4 (mycotoxin produced
30 by *Ustilago spp*);

31

1 Figure 21 is a graph showing the dose-dependent
2 effect of KP4 on the $[Ca^{2+}]_c$ response to 5 mM
3 external $CaCl_2$ (results represent mean \pm SE);
4

5 Figure 22a is a graph showing the effect of known
6 antifungal drugs on $[Ca^{2+}]_c$ in *Aspergillus nidulans*;
7

8 Figure 22b is a graph showing the effect of known
9 antifungal drugs on $[Ca^{2+}]_c$ in *Aspergillus niger*;
10

11 Figure 22c is a graph showing the effect of known
12 antifungal drugs on $[Ca^{2+}]_c$ in *Aspergillus awamori*;
13 and
14

15 Figure 23 is a graph showing the effect of
16 amphotericin B on $[Ca^{2+}]_c$ (results represent mean \pm
17 SE).
18

19 Figure 24 shows a graph showing the effect of Cr^{6+} (5
20 min preincubation) on aequorin light emission in
21 response to the addition of external $CaCl_2$ (5 mM).
22 Results represent mean \pm SE.
23

24 Figure 25 shows a graph showing the effect of Cr^{6+} (5
25 min preincubation) on $[Ca^{2+}]_c$ in response to the
26 addition of external $CaCl_2$ (5 mM). Results represent
27 mean \pm SE.
28

29 General cytotoxicity

30 - pure chemicals and chemical mixtures can be tested
31 for their toxicity using aequorin-expressed fungi.

32 Procedure:

- 1 i. Add compound(s) of interest to fungus
- 2 ii. Monitor $[Ca^{2+}]_c$ for 5 min
- 3 iii. Then stimulate fungus with mechanical
- 4 perturbation, hypo-osmotic, hyper-
- 5 osmotic shock
- 6 iv. Monitor $[Ca^{2+}]_c$ for further 5 min

7
8 The parameter to be assessed is $[Ca^{2+}]_c$ final resting
9 level. If $[Ca^{2+}]_c$ resting level is still elevated
10 more then 50% after the 11 min measurements the
11 compound(s) are toxic. The level of toxicity can be
12 assessed by subsequent monitoring of $[Ca^{2+}]_c$ for
13 several hours. The longer the $[Ca^{2+}]_c$ concentration
14 is out of normal the more toxic the compound(s) are.
15 This way there is no need for complicated software
16 and this type of approach is ideally suitable for
17 binary answer, based on 1 parameter.

18
19 Figure 16 shows a graph indicating the effect of 6
20 environmental samples on $[Ca^{2+}]_c$. The graph
21 indicates that sample 006 is toxic as the $[Ca^{2+}]_c$
22 final resting level is increased by more than 150%
23 compared with the control.

24
25 Another parameter for the analysis of general
26 toxicity is the total amount of $[Ca^{2+}]_c$ emitted.
27 Based on this parameter it is very easy to build
28 dose response curves (see Fig. 21).

29

30 **High information multiparameters analysis**

31 - In cases when the binary answer is not sufficient
32 aequorin-based biosensor can produce much more

1 detailed data characterising not only the general
2 cytotoxicity but also penetrability (by analysing
3 the time between administration of the compound to
4 the point when $[Ca^{2+}]_c$ starts to increase) and modes-
5 of-action of the compounds (by comparing the profile
6 of $[Ca^{2+}]_c$ changes of the compound(s) of interest to
7 the library of profiles). If the mode-of-action of
8 the compound(s) of interest is unique and unknown
9 than the present invention can suggest whether the
10 compound(s) causes the permeabilization of the
11 membrane, opening of ion channels or the alteration
12 in behaviour of Ca^{2+} carriers. This approach is
13 ideally suitable for analysis of combinations of
14 compounds.

15
16 This approach can be used for both pollutants
17 monitoring as previously described but also for the
18 analysis of drug toxicity. e.g. ibuprofen and
19 verapamil. Figures 17 and 18 show the effect of
20 ibuprofen and verapamil analogues on the $[Ca^{2+}]_c$ and
21 the table shown in Figure 19 further summarises the
22 profiles of the ibuprofen and verapamil analogues.

23 **Profiling compounds of interest and creating the**
24 **libraries of fingerprints of compounds**

25 - The present invention is ideally suitable for
26 creating the library of profiles for certain
27 substances. These profiles are unique to a compound
28 with the particular mode-of-action. Also they are
29 unique to the strain of fungus used, which allows
30 creating very details and reproducible fingerprint
31 of a particular compound using the present
32 invention. The profiles can be created with

1 different physico-chemical stimuli (e.g. mechanical
2 perturbation, hypo-osmotic, hyper-osmotic shock,
3 cold shock, heat shock, pH shock). These
4 fingerprints can be programmed into the software and
5 any compounds or mixtures of interest can be
6 screened to match the desired fingerprint.

7

8 Procedure to create the fingerprint:

- 9 • Monitor initial $[Ca^{2+}]_c$ resting level for 1
10 min
11 • Add compound(s) of interest to fungus
12 • Monitor $[Ca^{2+}]_c$ for 5 min
13 • Then stimulate fungus with mechanical
14 perturbation, hypo-osmotic, hyper-osmotic
15 shock
16 • Monitor $[Ca^{2+}]_c$ for further 5 min
17 • Based on the data obtained the following
18 parameters can be quantified for each
19 $[Ca^{2+}]_c$ increase occurring during the
20 experiment.

21 Lag time

22 Rise time

23 Amplitude absolute

24 Amplitude relative

25 Length of transient (LT_{20} , LT_{50} ,
26 LT_{80})

27 Initial $[Ca^{2+}]_c$ level

28 Final $[Ca^{2+}]_c$ resting level

29 Recovery time

30 Total concentration of $[Ca^{2+}]_c$

- 1 • Above 6 steps can be performed on
- 2 different strains
- 3 • Compound can be tested at different
- 4 concentrations

5

6 Considering the nature of the experiment the minimum
7 number of parameters produced by one compound at a
8 particular concentration on one fungal strain is
9 equal 22.

10

11 **Analysis of food and drink products for the presence** 12 **of mycotoxins**

13 - Fungi transformed with aequorin gene can be also
14 used for the analysis of food and drink products for
15 the presence of mycotoxins since these toxins affect
16 $[Ca^{2+}]_c$. Examples of such effects are shown in Figure
17 20 where the effects of cyclopiazonic acid (CPA) and
18 KP4 (mycotoxins produced by *Ustilago spp*) are
19 summarised.

20

21 **Cosmetics safety testing**

22 - Since EU regulations forbid the use of animal
23 testing for cosmetics industry the manufacturers are
24 looking at the alternative methods to assess the
25 effect of new products. As the present invention is
26 ideally suited for analysis of not only pure
27 compounds but also their mixtures, it could be used
28 for analysis of the safety of novel cosmetic
29 products. The present invention is also ideal for a
30 long term monitoring of the effects of compounds (up
31 to 96 h), which therefore allows analysis of the
32 longer-term toxicity than bacterial biosensors. The

1 present invention is also suitable for use on
2 different substrates such as solid and liquid
3 supports.

4

5 **Identification of different fungal strains**

6 - It has been found that each particular compound
7 produces a different fingerprint when added to
8 different fungal species. This can be used to
9 diagnose the unknown fungus.

10

11 **Procedure:**

- 12 • The fungus can be either transformed with
13 the recombinant aequorin gene or can be
14 injected with the active aequorin.
15 • Then this fungus can be subjected to a
16 range of the antifungal drugs, profiles of
17 which have already been created.
18 • Obtained profiles can be compared with the
19 library of the fingerprints and this way
20 the fungal species can be identified.

21 Figures 22a, b and c show that 5 known antifungal
22 drugs (ketoconazole, clotrimazole, amphotericin B,
23 nystatin and filipin) caused a different $[Ca^{2+}]_i$
24 response in 3 different species of *Aspergillus* (*A.*
25 *nidulans*, *A. niger*, *A. awamori*).

26

27 **Optimisation of the current antifungal treatments**

28 - In order to administer drugs in the best possible
29 way it is important to determine no effect
30 concentration and dose response curves, and
31 frequency for administration of drugs. Also, in
32 view of the developing resistance of fungus and

1 other eukaryotes to currently available drugs,
2 clinicians are looking into using combination of
3 drugs. The present invention is ideally suitable for
4 such studies.

5

6 **Identification of compounds which would prevent**
7 **fungal growth on plastics, metals and other**
8 **materials**

9 - Since the present invention is suitable for long
10 term measurements it is possible to monitor the
11 development and growth of fungi on different
12 materials and plastics treated with different
13 agents. It is possible to monitor the state of
14 fungal physiology by subjecting the organism to
15 different physico chemical treatments and analysis
16 of the profiles obtained.

17

18 Although the invention has been particularly shown
19 and described with reference to particular examples,
20 it will be understood by those skilled in the art
21 that various changes in the form and details may be
22 made therein without departing from the scope of the
23 present invention.